Practitioner's Docket No. 55107

CHAPTER II

TRANSMITTAL LETTER TO THE UNITED STATES ELECTED OFFICE (EO/US) (ENTRY INTO U.S. NATIONAL PHASE UNDER CHAPTER II)

PCT/JP99/02305	30 April 1999	30 April 1998
INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED

NICOTIANAMINE SYNTHASE AND GENE ENCODING THE SAME
TITLE OF INVENTION

Satoshi MORI, Kyoko HIGUCHI, Kazuya SUZUKI, Naoko NISHIZAWA and Hiromi NAKANISHI
APPLICANTS

Box PCT
Assistant Commissioner for Patents
Washington D.C. 20231
ATTENTION: EO/US

NOTE: To avoid abandonment of the application, the applicant shall furnish to the USPTO, not later than 20 months from the priority date: (1) a copy of the international application, unless it has been previously communicated by the International Bureau or unless it was originally filed in the USPTO; and (2) the basic national fee (see 37 C.F.R. § 1.492(a)). The 30-month time limit may not be extended. 37 C.F.R. § 1.495.

WARNING: *Where the items are those which can be submitted to complete the entry of the international application into the national phase are subsequent to 30 months from the priority date the application is still considered to be in the international state and if mailing procedures are utilized to obtain a date the express mail procedure of 37 C.F.R. §1.10 must be used (since international application papers are not covered by an ordinary certificate of mailing - See 37 C.F.R. §1.8.*

NOTE. Documents and fees must be clearly identified as a submission to enter the national state under 35 USC 371 otherwise the submission will be considered as being made under 35 USC 111. 37 C.F.R. § 1.494(f).

CERTIFICATION UNDER 37 C.F.R. § 1.10*
*(Express Mail label number is **mandatory.**)*
(Express Mail certification is optional)

I hereby certify that this paper, along with any document referred to, is being deposited with the United States Postal Service on this date October 30, 2000, in an envelope as "Express Mail Post Office to Addressee," mailing Label Number **EK929188236US**, addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

Deanna M. Rivernider
(type or print name of person mailing paper)

Deanna M. Rivernider
Signature of person mailing paper

WARNING: *Certificate of mailing (first class) or facsimile transmission procedures of 37 C.F.R § 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence.*

****WARNING:*** *Each paper or fee filed by "Express Mail" **must** have the number of the "Express Mail" mailing label placed thereon prior to mailing. 37 C.F.R § 1.10(b).*
*"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will **not** be granted on petition" Notice of Oct. 24, 1996, 60 Fed Reg 56,439, at 56,442.*

1. Applicant herewith submits to the United States Elected Office (EO/US) the following items under 35 U.S.C. 371:

   a. [X] This express request to immediately begin national examination procedures (35 U.S.C. 371(f)).

   b. [X] The U.S. National Fee (35 U.S.C. 371(c)(1)) and other fees (37 C.F.R. § 1.492) as indicated below:

2. Fees

CLAIMS FEE [X]*	(1) FOR	(2) NUMBER FILED	(3) NUMBER EXTRA	(4) RATE	(5) CALCULATIONS
	TOTAL CLAIMS	27- 20 =	7	x $ 18.00 =	$126.00
	INDEPENDENT CLAIMS	2 - 3 =	0	x $ 78.00 =	$0
	MULTIPLE DEPENDENT CLAIM(S) (if applicable) + $260.00				$260.00
BASIC FEE**	[ ] U.S. PTO WAS INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY Where an International preliminary examination fee as set forth in § 1.482 has been paid on the international application to the U.S. PTO: [ ] and the international preliminary examination report states that the criteria of novelty, inventive step (non-obviousness) and industrial activity, as defined in PCT Article 33(2) to (4) have been satisfied for all the claims presented in the application entering the national stage (37 CFR 1.492(a)(4)) ..... $96.00 [ ] and the above requirements are not met (37 CFR 1.492(a)(1)) ..... $670.00 [X] U.S. PTO WAS NOT INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY Where no international preliminary examination fee as set forth in § 1.482 has been paid to the USPTO, and payment of an international search fee as set forth in § 1.445(a)(2) to the U.S. PTO: [ ] has been paid (37 CFR 1.492(a)(2)) ..... $760.00 [ ] has not been paid (37 CFR 1.492(a)(3)) ..... $970.00 [X] where a search report on the international application has been prepared by the European Patent Office or the Japanese Patent Office (37 CFR 1.492(a)(5)) $860.00				$860.00
	Total of above Calculations				= $1,246.00
SMALL ENTITY	Reduction by ½ for filing by small entity, if applicable. Affidavit must be filed. (note 37 CFR 1.9, 1.27, 1.28)				- $
	Subtotal				$1,246.00
	Total National Fee				$1,246.00
	Fee for recording the enclosed assignment document $40.00 (37 CFR 1.21(h)). (See Item 13 below). See attached "ASSIGNMENT COVER SHEET".				$
TOTAL	Total Fees enclosed				$1,246.00

*See attached Preliminary Amendment Reducing the Number of Claims.

i. [X] A check in the amount of $1,246.00 to cover the above fees is enclosed.

ii. [ ] Please charge Account No. _____ in the amount of $ _____.
A duplicate copy of this sheet is enclosed.

***WARNING:* *"To avoid abandonment of the application the applicant shall furnish to the United States Patent and Trademark Office not later than the expiration of 30 months from the priority date. * * * (2) the basic national fee (see § 1.492(a)). The 30-month time limit may not be extended." 37 C.F.R. § 1.495(b).*

WARNING: *If the translation of the international application and/or the oath or declaration have not been submitted by the applicant within thirty (30) months from the priority date, such requirements may be met within a time period set by the Office. 37 C.F.R. § 1.495(b)(2). The payment of the surcharge set forth in § 1.492(e) is required as a condition for accepting the oath or declaration later than thirty (30) months after the priority date. The payment of the processing fee set forth in § 1.492(f) is required for acceptance of an English translation later than thirty (30) months after the priority date. Failure to comply with these requirements will result in abandonment of the application. The provisions of § 1.136 apply to the period which is set. Notice of Jan. 3, 1993, 1147 O.G. 29 to 40.*

3. [X] A copy of the International application as filed (35 U.S.C. 371(c)(2)):

NOTE: *Section 1.495 (b) was amended to require that the basic national fee and a copy of the international application must be filed with the Office by 30 months from the priority date to avoid abandonment "The International Bureau normally provides the copy of the international application to the Office in accordance with PCT Article 20. At the same time, the International Bureau notifies applicant of the communication to the Office. In accordance with PCT Rule 47.1, that notice shall be accepted by all designated offices as conclusive evidence that the communication has duly taken place. Thus, if the applicant desires to enter the national stage, the applicant normally need only check to be sure the notice from the International Bureau has been received and then pay the basic national fee by 30 months from the priority date." Notice of Jan. 7, 1993, 1147 O.G. 29 to 40, at 35-36. See item 14c below.*

a. [ ] is transmitted herewith.
b. [ ] is not required, as the application was filed with the United States Receiving Office.
c. [X] has been transmitted
   i. [X] by the International Bureau.
   Date of mailing of the application (from form PCT/IB/308): 11/11/99.
   ii. [ ] by applicant on _____.
   Date

4. [X] A translation of the International application into the English language (35 U.S.C. 371(c)(2)):
a. [X] is transmitted herewith.
b. [ ] is not required as the application was filed in English.
c. [ ] was previously transmitted by applicant on _____.
   Date
d. [ ] will follow.

5. [X] Amendments to the claims of the International application under PCT Article 19 (35 U.S.C. 371(c)(3)):

NOTE: *The Notice of January 7, 1993 points out that 37 C.F.R. § 1.495(a) was amended to clarify the existing and continuing practice that PCT Article 19 amendments must be submitted by 30 months from the priority date and this deadline may not be extended. The Notice further advises that: "The failure to do so will not result in loss of the subject matter of the PCT Article 19 amendments. Applicant may submit that subject matter in a preliminary amendment filed under section 1.121. In many cases, filing an amendment under section 1.121 is preferable since grammatical or idiomatic errors may be corrected." 1147 O.G. 29-40, at 36.*

a. [ ] are transmitted herewith.
b. [ ] have been transmitted
   i. [ ] by the International Bureau.

Date of mailing of the amendment (from form PCT/IB/308): _____.

ii. [ ] by applicant on _____.
Date

c. [X] have not been transmitted as

i. [X] applicant chose not to make amendments under PCT Article 19.
Date of mailing of Search Report (from form PCT/ISA/210): **06/07/99**

ii. [ ] the time limit for the submission of amendments has not yet expired. The amendments or a statement that amendments have not been made will be transmitted before the expiration of the time limit under PCT Rule 46.1.

6. [X] A translation of the amendments to the claims under PCT Article 19 (38 U.S.C. 371(c)(3)):

a. [ ] is transmitted herewith.
b. [ ] is not required as the amendments were made in the English language.
c. [X] has not been transmitted for reasons indicated at point 5(c) above.

7. [X] A copy of the international examination report (PCT/IPEA/409)

[X] is transmitted herewith.
[ ] is not required as the application was filed with the United States Receiving Office.

8. [ ] Annex(es) to the international preliminary examination report

a. [ ] is/are transmitted herewith.
b. [ ] is/are not required as the application was filed with the United States Receiving Office.

9. [ ] A translation of the annexes to the international preliminary examination report

a. [ ] is transmitted herewith.
b. [ ] is not required as the annexes are in the English language.

10. [X] An oath or declaration of the inventor (35 U.S.C. 371(c)(4)) complying with 35 U.S.C. 115

a. [ ] was previously submitted by applicant on _____.
Date

b. [ ] is submitted herewith, and such oath or declaration

i. [ ] is attached to the application.
ii. [ ] identifies the application and any amendments under PCT Article 19 that were transmitted as stated in points 3(b) or 3(c) and 5(b); and states that they were reviewed by the inventor as required by 37 C.F.R. 1.70.
iii. [X] will follow.

II. Other document(s) or information included:

11. [X] An International Search Report (PCT/ISA/210) or Declaration under PCT Article 17(2)(a):

a. [X] is transmitted herewith.
b. [ ] has been transmitted by the International Bureau.
Date of mailing (from form PCT/IB/308): _____.
c. [ ] is not required, as the application was searched by the United States International Searching Authority.

d. [ ] will be transmitted promptly upon request.
e. [ ] has been submitted by applicant on _____. Date

12. [X] An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98:
a. [X] is transmitted herewith.
Also transmitted herewith is/are:
[X] Form PTO-1449 (PTO/SB/08A and 08B).
[X] Copies of citations listed.
b. [ ] will be transmitted within THREE MONTHS of the date of submission of requirements under 35 U.S.C. 371(c).
c. [ ] was previously submitted by applicant on _____. Date

13. [X] An assignment document is transmitted herewith for recording.

A separate [ ] "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or [X] FORM PTO 1595 is also attached.

14. [X] Additional documents:
a. [X] Copy of request (PCT/RO/101)
b. [X] International Publication No. WO99/57249
i. [X] Specification, claims and drawing
ii. [ ] Front page only
c. [ ] Preliminary amendment (37 C.F.R. § 1.121)
d. [X] Other

Preliminary Amendment, Forms PCT/ISA/220, PCT/IB/304, PCT/IB/308, (Written Opinion), PCT/IB/301, PCT/IPEA/416,

15. [X] The above checked items are being transmitted
a. [X] before 30 months from any claimed priority date.
b. [ ] after 30 months.

16. [ ] Certain requirements under 35 U.S.C. 371 were previously submitted by the applicant on _____, namely:

AUTHORIZATION TO CHARGE ADDITIONAL FEES

WARNING: *Accurately count claims, especially multiple dependent claims, to avoid unexpected high charges if extra claims are authorized.*

NOTE: "A written request may be submitted in an application that is an authorization to treat any concurrent or future reply, requiring a petition for an extension of time under this paragraph for its timely submission, as incorporating a petition for extension of time for the appropriate length of time. An authorization to charge all required fees, fees under § 1.17, or all required extension of time fees will be treated as a constructive petition for an extension of time in any concurrent or future reply requiring a petition for an extension of time under this paragraph for its timely submission. Submission of the fee set forth in § 1.17(a) will also be treated as a constructive petition for an extension of time in any concurrent reply requiring a petition for an extension of time under this paragraph for its timely submission." 37 C.F R. § 1 136(a)(3).

NOTE: "Amounts of twenty-five dollars or less will not be returned unless specifically requested within a reasonable time, nor will the payer be notified of such amounts; amounts over twenty-five dollars may be returned by check or, if requested, by credit to a deposit account." 37 C.F.R. § 1.26(a).

[X] The Commissioner is hereby authorized to charge the following additional fees that may be required by this paper and during the entire pendency of this application to Account No. **04-1105**.

[X] 37 C.F.R. 1.492(a)(1), (2), (3), and (4) (filing fees)

WARNING: *Because failure to pay the national fee within 30 months without extension (37 C.F.R. § 1.495(b)(2)) results in abandonment of the application, it would be best to always check the above box.*

[X] 37 C.F.R. 1.492(b), (c) and (d) (presentation of extra claims)

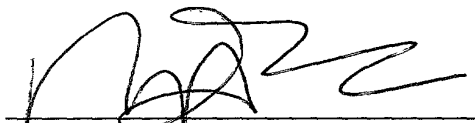
NOTE: Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must only be paid or these claims cancelled by amendment prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency (37 C.F.R. § 1.492(d)), it might be best not to authorize the PTO to charge additional claim fees, except possible when dealing with amendments after final action

[X] 37 C.F.R. 1.17 (application processing fees)
[X] 37 C.F.R. 1.17(a)(1)-(5)(extension fees pursuant to § 1.136(a).
[ ] 37 C.F.R. 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 C.F.R. 1.311(b))

NOTE: Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of a Notice of Allowance, the issue fee will be automatically charged to the deposit account at the time of mailing the notice of allowance. 37 C.F.R. § 1.311(b).

NOTE: 37 C F.R. 1.28(b) requires "Notification of any change in loss of entitlement to small entity status must be filed in the application . . prior to paying, or at the time of paying . . . issue fee." From the wording of 37 C F.R. § 1.28(b): (a) notification of change of status must be made even if the fee is paid as "other than a small entity" and (b) no notification is required if the change is to another small entity.

[ ] 37 C.F.R. § 1.492(e) and (f) (surcharge fees for filing the declaration and/or filing an English translation of an International Application later than 30 months after the priority date).



SIGNATURE OF PRACTITIONER

Reg. No.: 33,860

Peter F. Corless
(type or print name of practitioner)

EDWARDS & ANGELL, LLP
Dike, Bronstein, Roberts & Cushman, IP Group

Tel. No.: (617) 523-3400

130 Water Street
P.O. Address

Customer No.:

Boston, MA 02109

NICOTIANAMINE SYNTHASE AND GENE ENCODING THE SAME

Technical Field

The present invention relates to a nicotianamine synthase involved in the mugineic acid biosynthetic pathway, the amino acid sequence thereof, a gene encoding the same, a vector, a process for producing nicotianamine by using the same, plants transformed by the gene encoding the nicotianamine synthase, and an antibody against the nicotianamine synthase.

Background Art

Graminaceous plants that absorb by chelating the insoluble state Fe(III) in soil using mugineic acid and adopt so called the Strategy-II mechanism of Fe acquisition secrete Fe chelators (phytosiderophores) from their roots to solubilize sparingly soluble Fe in the rhizosphere (Roemheld, 1987). The amount of the secreted phytosiderophores increases under Fe-deficiency stress. The mugineic acid family is the only examples of phytosiderophores known so far (Takagi, 1976). Tolerance to Fe deficiency in graminaceous plants is thought to depend on a quantity of mugineic acid family secreted by plants (Takagi et al. 1984, Roemheld and Marschner 1986, Marschner et al. 1987, Mori et al. 1987, Kawai et al. 1988, Mori et al. 1988, Mihashi and Mori 1989, and Shingh et al. 1993).

The biosynthetic pathway of mugineic acid in plants is shown in Fig. 1. S-adenosylmethionine is synthesized from methionine by S-adenosylmethionine synthase. Subsequently, three molecules of S-adenosylmethionine are combined to form one molecule of nicotianamine by nicotianamine synthase. The generated nicotianamine is then converted to 3"-keto acid by nicotianamine aminotransferase,

and 2'-deoxymugineic acid is synthesized by the subsequent action of a reductase. A further series of hydroxylation steps produces the other mugineic acid derivatives including mugineic acid from the deoxymugineic acid (Mori and Nishizawa 1987, Shojima et al. 1989, Shojima et al. 1990 and Ma and Nomoto 1993).

A compound in Fig. 1, a compound in the lower right, wherein R_1 and R_2 are hydrogen and R_3 is hydroxyl, is mugineic acid. A compound wherein R_1 is hydrogen and R_2 and R_3 are hydroxyl, is 3-hydroxymugineic acid. Also a compound wherein R_2 is hydrogen and R_1 and R_3 are hydroxyl, is 3-epihydroxymugineic acid.

Three S-adenosylmethionine synthase genes were isolated from barley roots, but these genes were not induced by Fe deficiency (Takizawa et al. 1996). A gene Ids3, which is obtained from the barley by differential screening, is suspected to be a gene, which converts deoxymugineic acid to mugineic acid by hydroxylation and is strongly induced by Fe-deficiency (Nakanishi et al. 1993). Further, nicotianamine aminotransferase was purified and isolated from Fe-deficient barley roots, and two nicotianamine aminotransferase genes, Naat-A and Naat-B, were isolated (Takahashi et al. 1997). Naat-A expression was induced in Fe-deficient roots.

The synthesis of nicotianamine from S-adenosylmethionine is similar to polyamine synthesis from decaroboxy-S-adenosylmethionine. In contrast to polyamine synthase, however, nicotianamine synthase catalyzes the combination of three S-adenosylmethionine molecules and the azetidine ring formation at the same time (Fig. 1). Such the nicotianamine synthase is a novel type of enzyme. Previously, we reported the partial purification of nicotianamine synthase from the roots of Fe-deficient barley and expression pattern of the activity (Higuchi et al. 1994, Higuchi et al. 1995, Kanazawa et al. 1995, Higuchi et al. 1996a and Higuchi et al. 1996b). Since nicotianamine synthase is easily decomposed during extraction and purification, it has

been difficult to purify sufficient quantities for amino acid sequencing.

The present invention has an object to provide a plant, especially graminaceous plant, highly tolerant to Fe-deficiency, as a result of isolating and purifying a nicotianamine synthase, being cloned the gene of this enzyme, determining the base sequence and amino acid sequence thereof, and using said enzyme.

Disclosure of Invention

The present invention relates to a nicotianamine synthase shown in SEQ ID NO: 1 comprising amino acid sequence shown in SEQ ID NO: 1, or amino acid sequence having deletion in a part thereof, being substituted by the other amino acids or being added with the other amino acids.

The present invention relates to the gene encoding said amino acid sequence of nicotianamine synthase.

The present invention also relates to a vector comprising containing said gene, and a transformant transformed by the said vector.

The present invention relates to a process for production of nicotianamine using the said transformant.

The present invention further relates to plants, especially graminaceous plants, to which said gene is introduced, and fruits obtained by growing said plants.

The present invention relates to a process for extraction of said nicotianamine synthase in the presence of thiol protease inhibitor, preferably E-64.

Further, the present invention relates to an antibody against said nicotianamine synthase.

Brief Description of Drawing

Fig. 1 shows the biosynthetic pathway of mugineic acid family.

Fig. 2 shows a comparison of nicotianamine synthase purification from Fe-dependent and control barley roots.

Fig. 3 shows a preparative SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis, hereinafter designates as SDS-PAGE) around 30 - 35 kDa. The horizontal bar indicates relative enzyme activity detected from the gels.

Fig. 4 shows elution pattern of nicotianamine synthase activity from the gel-filtration column.

The large closed circles (●) indicates enzyme activity.

Fig. 5 shows a comparison with a six partial amino acid sequence determined by nicotianamine synthase originated from barley and similar sequence of graminaceous plants obtained by computer search of the database. Identical amino acid residue is shown in " : " .

Fig. 6 shows full length of HvNAS1 cDNA and amino acid sequence deduced therefrom. The underlined sequences indicate the identical partial amino acid sequences of fragments in the above Fig. 5. Numbers of the nucleotide sequence are indicated to the right of each row. Amino acid numbers are indicated on the left of each row.

Fig. 7 shows comparison of the deduced amino acid sequences of the above 7 cDNA obtained from barley. Asterisks " * " indicates identical amino acid residues in all sequences.

Fig. 8 shows results of thin layer chromatographic (TLC) analysis of nicotianamine synthase activity obtained from E. coli crude extract expressing a fused protein of maltose binding protein - HvNAS1.

Fig. 9 shows Northern - hybridization analysis of HvNAS1 as a probe.

Fig. 10 shows Southern - hybridization analysis of HvNAS1 as a probe.

Fig. 11 shows Western-blot analysis of crude enzyme used for detection of nicotianamine synthase activity.

Fig. 12 shows Western-blot analysis of total protein extracted by trichloroacetic acid/acetone.

Fig. 13 shows comparison of nicotianamine synthase purification from Fe-deficient barley and control barley after DEAE-Sepharose FF.

Fig. 14 shows comparison of nicotianamine synthase purification from Fe-deficient barley and control barley after Ether Toyopearl 650M.

Fig. 15 shows results of thin layer chromatographic (TLC) analysis of nicotianamine synthase activity obtained from E. coli crude extract expressing a fused protein of maltose binding protein - OsNAS1.

Fig. 16 shows Northern - hybridization analysis of OsNAS1 as a probe.

Fig. 17 shows results of thin layer chromatographic (TLC) analysis of nicotianamine synthase activity obtained from E. coli crude extract expressing a fused proteins of maltose binding protein - AtNAS1, AtNAS2 or AtNAS3.

Fig. 18 shows results of RT-PCR of total RNA extracted from the aboveground parts and roots of Arabidopsis thaliana. Right group indicates positive control.

Best Mode for Carrying Out the Invention

We have tried to isolate nicotianamine synthase (Higuchi et al. Plant & Soil, Vol. 165, p. 173-179, 1994), and since nicotianamine synthase was easily decomposed and was difficult to isolate and purify, we were unable to obtain sufficient amounts of protein to determine its partial amino acid sequence. Subsequently, it was found that a

thiol protease inhibitor E-64 (hereinafter designates as E-64) was very effective in suppressing degradation of nicotianamine synthase (Higuchi et al. Plant & Soil, Vol. 178, p. 171 - 177, 1996 a).

In the present invention, as a result that frozen roots were crushed to a fine powder in liquid N_2 and then rapidly homogenized with buffer containing 0.1 mM thiol protease inhibitor E-64, nicotianamine synthase protein could be isolated and its gene could also be isolated.

Further, the enzyme of the present invention recovered its activity by removal of SDS after SDS-PAGE treatment, but the rate of recovery was very low (Higuchi et al. Plant & Soil, Vol. 165, p. 173-179, 1994). Consequently, degree of purification should be increased up before treatment of SDS-PAGE. Then the column chromatography procedures were further improved.

We have also found that the enzyme of the present invention is relatively hydrophobic and a buffer containing a mild surface active agent CHAPS increased the rate of recovery. Several ion-exchange chromatography carriers were tested, and DEAE-Sepharose FF and DEAE Sephacel were found to be the most effective. In addition to TSK gel Butyl Toyopearl, another hydrophobic chromatography carrier, TSK gel Ether Toyopearl 650M, effectively removed impurities of the 30 - 35 kDa.

The enzyme of the present invention has been reported that it was the peptide of 30 - 35 kDa, the activity of which was recovered by removing SDS after SDS-PAGE treatment, and the activity was detected as a broad molecular weight range of 30 - 35 kDa (refer to Fig. 3). Fig. 3 shows a result of preparative SDS-PAGE in the fractions showing enzyme activity. SDS-PAGE was carried out using 11% acrylamide slab gels. A portion of the gel was stained with Coomassie brilliant blue and the rest of the gel was stained with Cu. The gel containing proteins between 30 - 35 kDa in size was cut

into seven fragments (indicated by the short lines). The thick bars in Fig. 3 indicate relative enzymatic activities detected from each gel fragment.

In order to identify nicotianamine synthase peptide from the proteins having these molecular weights, the peptides, which were contained in the nicotianamine synthase fractions, purified from Fe-deficient and control barley roots were compared using SDS-PAGE. From each barley root 200 g, the present enzyme was purified according to the method described in example 3 hereinbelow.

The enzyme activity of the control was a quarter of the Fe-deficient roots.

The peptide composition of the active enzyme fraction from each purification step of the present enzyme was analyzed and compared by SDS-PAGE, and results are shown in Fig. 2, Fig. 13 and Fig. 14. Fig. 2, Fig. 13 and Fig. 14 show comparison with the active fraction from the purification step of Fe-deficient barley roots 200 g [in the figure, shown with (-)], and the active fraction from the purification step of the control barley roots 200 g [in the figure, shown with (+)]. SDS-PAGE was carried out using 12.5% acrylamide slab gels (Laemmli, Nature Vol. 227, p. 680-685, 1970). Gels were stained with Coomassie brilliant blue. Fig. 2 shows a step before DEAE-Sepharose. The upper row shows enzyme from Fe-deficient barley roots and the lower row shows enzyme from control roots. In each lane, lanes 1, crude extract, 200 μg of protein; lanes 2, after Butyl Toyopearl 650M, 100 μg of protein; lanes 3, after hydroxyapatite, 20 μg of protein; and lanes 4, after Butyl Toyopearl 650M, 15 μg of protein, are shown.

Fig. 13 shows after DEAE-Sepharose FF, each lane, 25 μg of protein. Fig. 14 shows after Ether Toyopearl 650M; in which left shows inactive fraction, and right shows active fraction, and 1/25 of each fraction is electrophoresed.

As a result, almost no difference was observed in both Fe-deficient and control roots before DEAE-Sepharose step (refer to Fig. 2). After the DEAE-Sepharose step it

became clear that the 30- and 31-kDa peptides were induced by Fe-deficiency (refer to Fig. 13). After the Ether Toyopearl step, the 31 kDa peptide was eliminated from the active fraction. The 32 and 33 kDa peptides were found to be newly induced by Fe-deficiency (refer to Fig. 14). Activities were detected from the 32 and 33 kDa peptides, but no activity was detected from 30 kDa peptide (refer to Fig. 3).

Molecular weight of the enzyme of the present invention was determined by gel-filtration.

Estimated molecular weight of nicotianamine synthase by gel-filtration was reported to be 40,000 - 50,000 (Higuchi et al. Plant & Soil, Vol. 165, p. 173-179, 1994). But this did not correspond with the value estimated by SDS-PAGE.

In the present study, the buffer containing CHAPS effectively increased the resolution and molecular weight of the present enzyme was estimated to be 35,000 (refer to Fig. 4). this corresponds well to the value estimated by SDS-PAGE.

Fig. 4 shows elution pattern of nicotianamine synthase from the gel-filtration column. The black circles (●) indicate the enzyme activity and the solid line indicates absorption at 280 nm. The active fraction after hydroxyapatite chromatography was applied to a Sephacryl S300HR (Pharmacia) column (1.5 cm × 71 cm, 125 ml), equilibrated with developing buffer (50 mM Tris, 1 mM EDTA, 0.1 M KCl, 0.05% CHAPS, 0.1 mM p-APMSF and 3 mM DTT, pH 8.0). Molecular weight markers used were thyroglobulin (Mr 670,000), γ-globulin (Mr 158,000), ovalbumin (Mr 44,000), and myoglobin (Mr 17,000). The linear flow was 10 cm/hour.

Partial amino acid sequence was determined from purified nicotianamine synthase.

The above explained 30 kDa, 32 kDa and 33 kDa peptides were purified from 1 kg of Fe-deficient barley roots by using a method in example 3 hereinbelow. These

were partially degraded using a method in example 4 hereinbelow. Although 32- and 33-kDa peptides could not be completely separated from each other, these might have similar sequence or 32 kDa peptide was presumed to be the degradation product of 33 kDa peptide, and both of them were degraded in together.

The determined partial amino acid sequences indicated that these peptides were very similar in each other (Fig. 5). Further, since the molecular weights of the 33 kDa and 32 kDa (1) fragments had almost unchanged molecular weight as compared with before degradation, this sequence might be N-terminal region of the present enzyme. As a result of computer search of the database, a gene of unknown function having very similar sequence to these sequences was found to exist in Oryza sativa and Alabidopsis thaliana. Especially, EST-cDNA clones D23792 and D24790 of Oryza sativa were very similar with 80.0% identity in a 33-amino acid overlap in the former and 68.4% identity in a 19-amino acid overlap in the latter (Fig. 5).

Fig. 5 shows a comparison with a six partial amino acid sequence determined by nicotianamine synthase originated from barley and similar sequence of graminaceous plants obtained by computer search of the database. Identical amino acid residue is shown in ":". The part of nucleotide sequences indicated by the arrows was applied for the sequences of primer used in PCR.

Cloning and nucleotide sequences of cDNA clones encoding nicotianamine synthase were performed and determined.

PCR amplification of total cDNA prepared from Fe-deficient barley roots using degenerate primers designed from the partial amino acid sequence obtained from the method explained hereinbefore was performed, but the objective DNA could not amplified. Then the primers having single nucleotide sequence (shown by arrows in Fig. 5) from sequences of Oryza sativa, D23792 and D24790, were synthesized and

PCR amplification was performed. The 205 bp fragment was amplified by PCR using NF and NR primers and the 274 bp fragment was amplified by PCR using IF and IR primers, and these contained the objective sequences. A cDNA library prepared using poly (A)$^+$ RNA from Fe-deficient barley roots was screened and 19 positive clones using the 205 bp fragment probe and 88 positive clones using the 274 fragment bp probe were obtained.

Among the thus obtained clones, the clone designated as HvNAS1, contained a translated region of 985 bp and amino acid sequence deduced therefrom was 328 amino acids residue, with deduced molecular weight of 35,144. This corresponded well with the value estimated by SDS-PAGE and gel-filtration. The partial amino acid sequences of the 32 kDa and 33 kDa peptides were included totally in HvNAS1 (Fig. 6).

Fig. 6 shows full length of HvNAS1 cDNA and amino acid sequence deduced therefrom. The underlined sequences indicate the identical partial amino acid sequences of fragments in the above Fig. 5. Numbers of the nucleotide sequence are indicated to the right of each row. Amino acid numbers are indicated on the left of each row.

The predicted pI of 5.2 matched the value estimated by native isoelectric focusing electrophoresis well. The six clones having very similar sequence other than HvNAS1, i.e. HvNAS2, HvNAS3, HvNAS4, HvNAS5, HvNAS6 and HvNAS7, were also obtained (Table 1, Fig. 7).

Fig. 7 shows comparison of the deduced amino acid sequences of the above 7 cDNA obtained from barley. Asterisks "*" indicates identical amino acid residues in all sequences.

The nucleotide sequences of these clones are shown in SEQ ID NO: 2 (HvNAS1), SEQ ID NO: 4 (HvNAS2), SEQ ID NO: 6 (HvNAS3), SEQ ID NO: 8

(HvNAS4), SEQ ID NO: 10 (HvNAS5), SEQ ID NO: 12 (HvNAS6) and SEQ ID NO: 14 (HvNAS7), respectively. The amino acid sequences of these amino acid sequences are shown in SEQ ID NO: 1 (HvNAS1), SEQ ID NO: 3 (HvNAS2), SEQ ID NO: 5 (HvNAS3), SEQ ID NO: 7 (HvNAS4), SEQ ID NO: 9 (HvNAS5), SEQ ID NO: 11 (HvNAS6) and SEQ ID NO: 13 (HvNAS7), respectively.

Table 1 Properties of nas clones

Clone	Number of Amino Acid Residues	Molecular Weight	pI	Identity to nas 1 (%)	Identity to nas 2 (%)	Identity to nas 4 (%)
HvNAS 1	328	35144	5.20	-		
HvNAS 2	336	35839	5.07	72	-	
HvNAS 3	336	36013	5.47	72	95	
HvNAS 4	330	35396	4.91	73	89	-
HvNAS 5	283	30148	5.22	61	61	59
HvNAS 6	329	35350	5.07	74	89	88
HvNAS 7	330	35244	4.98	70	86	91

The partial amino acid sequences determined from the 30 kDa peptide were all included in HvNAS5. The 5'- and 3'-non-translated regions of these clones were not similar with each other.

D23792 and D24790 similar to nicotianamine synthase of Oryzae sativa were found with about 80% identity to HvNAS1. AC003114 and AB005245 of Arbidopsis thaliana were found with about 45% identity to HvNAS1.

The obtained HvNAS1 protein was expressed in E. coli.

The PCR amplification of HvNAS1 ORF was cloned with vector pMAL-c2 to express HvNAS1 fused with C-terminal of maltose biding protein. The expression of fused protein is strongly induced by IPTG.

The crude extract was obtained from the transformed E. coli, and nicotianamine synthase activity was assayed in the state of the fused protein. The crude extract from the strain transformed with only the vector could not be detected the activity, whereas in case of inserted with HvNAS1 ORF, the activity was detected. Result is shown in Fig. 8.

Fig. 8 shows results of thin layer chromatographic (TLC) analysis of nicotianamine synthase obtained from E. coli crude extract expressing a fused protein of maltose binding protein - HvNAS1. In Fig. 8, lane 1: a standard nicotianamine synthase; lane 2: E. coli expressing maltose binding protein (SAM); and lane 3: E. coli expressing maltose binding protein - HvNAS1 fused protein.

Northern hybridization analysis conducted by the method described in example 7 hereinbelow indicated that this gene was strongly induced in Fe-deficient roots (Fig. 9). This coincides with expression pattern of the present enzyme activity (Higuchi et al. 1994). Fig. 9 shows a result of Northern hybridization analysis using HvNAS1 as a probe. Total RNA was extracted from after one week of Fe-deficient treatment and control barley leaves and roots, and in each lane, 5μg of RNA were electrophoresed.

Southern hybridization analysis of the barley genome DNA was performed according to the method described in example 8 hereinafter mentioned. Cutting of DNA with BamHI, EcoRI or HindIII produced plurality of fragments, however none of clones obtained at present could be digested by BamHI and EcoRI, consequently nicotianamine synthase gene might exist with multiple copies in genomes of barley and rice (Fig. 10).

Fig. 10 shows Southern - hybridization analysis of HvNAS1 as a probe. Genomic DNAs from barley and rice were digested with BamHI (lanes B), EcoRI (lanes

R) and HindIII (lanes H) and 10μg thereof were electrophoresed in each lane.

Further, using antigen prepared by the method described in example 9 hereinbelow, Western-blot analysis was performed according to the method described in example 10. It was found that the present enzyme protein was rapidly decomposed during the operation in the crude extract prepared for detecting the present enzyme activity (Fig. 11). The staining patterns coincided with the fact that the present enzyme activity was detected on the broad range between 30 - 35 kDa after SDS-PAGE (refer to Fig. 3).

Fig. 11 shows Western-blot analysis of crude enzyme used for detection of activity. SDS-PAGE was performed using 12.5% acrylamide slab gel. Protein 100μg was electrophoresed.

The crude extract obtained from denatured protein according to the method described in example 10 hereinbelow was detected as almost single band with 35 - 36 kDa (Fig. 12). This value coincided with the deduced value from the amino acid sequence.

Fig. 12 shows Western-blot analysis of total protein extracted by trichloroacetic acid/acetone. SDS-PAGE was performed using 12.5% acrylamide slab gel. Protein 100μg was electrophoresed. Proteins 200μg extracted from roots and proteins 500μg extracted from leaves were electrophoresed.

Western-blot analysis after 2-dimention electrophoresis reveals to detect several spots. This coincided with the fact of obtaining plurality of nicotianamine synthase gene. All spots were induced in Fe-deficient roots.

As a result that cDNA library from Fe-deficient rice roots poly (A) + RNA was screened using probes prepared by cutting HvNAS1 with restriction enzymes ApaLI and XhoI, 20 clones were obtained. These clones were divided into 3 types of clones

according to their sequences, and among them, only one type contains ORF full length, which was designated as OsNAS1. Nucleotide sequence of OsNAS1 is shown in SEQ ID NO: 16 and amino acid sequence is shown in SEQ ID NO: 15.

PCR amplification of OsNAS1 ORF was cloned with a vector pMAL-c2 to express a form fused with maltose binding protein C-terminal. The fused protein is strongly induced its expression by IPTG.

Crude extract from the transformed E. coli with the fused protein was obtained and nicotianamine synthase activity was assayed in the state of the fused protein. The same activity with HvNAS1 was detected. Result is shown in Fig. 15. Fig. 15 shows results of thin layer chromatographic (TLC) analysis of nicotianamine synthase obtained from E. coli crude extract expressing a fused protein of maltose binding protein - OsNAS1. In Fig. 15, lane 1: a standard nicotianamine (NA); lane 2: an extract from E. coli expressing maltose binding protein - OsNAS1 fused protein; and lane 3: an extract from E. coli expressing maltose binding protein - HvNAS1 fused protein.

Northern hybridization analysis conducted by the method described in example 7 hereinbelow indicated that in contrast to barley, the expression was induced in rice by Fe-deficient treatment not only in roots but also in leaves (Fig. 16). Fig. 16 shows a result of Northern hybridization analysis using OsNAS1 ORF as a probe. Total RNA was extracted from after two weeks of Fe-deficient treatment and control rice leaves and roots, and in each lane, 5 μg of RNA were electrophoresed.

Nucleotide sequence of Arabidopsis thaliana similar to HvNAS1 obtained by computer search of the database was used as a primer. PCR amplification for genome DNA of Arabidopsis thaliana resulted to obtain three nicotianamine synthase genes. These were designated as AtNAS1, AtNAS2 and AtNAS3.

Nucleotide sequence of these genes are shown in SEQ ID NO: 18 (AtNAS1), SEQ ID NO: 20 (AtNAS2) and SEQ ID NO: 22 (AtNAS3). These amino acid sequences are shown in SEQ ID NO: 17 (AtNAS1), SEQ ID NO: 19 (AtNAS2) and SEQ ID NO: 21 (AtNAS3).

AtNAS1, AtNAS2 and AtNAS3 ORF were amplified with PCR and were cloned with a vector pMAL-c2. Each of them was tried to be expressed in the form of fusing with maltose binding protein C-terminal. The expression of the fused protein was strongly induced by IPTG.

Crude extract from the transformed E. coli with the fused protein was obtained and nicotianamine synthase activity was assayed in the state of the fused protein. The activity was detected. Result is shown in Fig. 17. Fig. 17 shows results of TLC analysis of nicotianamine synthase activity obtained from E. coli crude extract expressing a fused protein of maltose binding protein - AtNAS. In Fig. 17, lanes 1: a standard nicotianamine (NA) and S-adenosylmethionine; lanes 2: an extract from E. coli expressing only maltose binding protein; lanes 3: an extract from E. coli expressing maltose binding protein - AtNAS1 fused protein; lanes 4: an extract from E. coli expressing maltose binding protein - AtNAS2 fused protein; and lanes 5: an extract from E. coli expressing maltose binding protein - AtNAS3 fused protein.

RT-PCR was conducted according to the method described in example 11 hereinbelow. It was found that AtNAS1 was expressed in the roots and the aboveground parts of Arabidopsis thaliana, whereas AtNAS2 was expressed neither in the roots nor in the aboveground parts, and AtNAS3 was expressed only in the roots (Fig. 18). In Fig. 18, lane M shows molecular weight marker. Gene expression was conducted in the aboveground parts, roots and positive controls. In the figure, lanes C: AtNAS1 and AtNAS2 ORF full length were amplified; lanes 1: AtNAS1 specific

amplification fragments; lanes 2: AtNAS2 specific amplification fragments; and lanes 3: AtNAS3 specific amplification fragments.

The amount of secreted mugineic acid is reported increased up to 20 mg mugineic acid/g roots dry weight/day (Takagi, 1993). Crude nicotianamine synthase activity detected by the present inventors was sufficient to fulfill it. Since the present enzyme proteins exist in more than several types and 30 kDa peptide without activity exists, it can be speculated that as a result of aggregation of these peptides, the constructed structure, which is preferable for binding with 3 molecules of S-adenosylmethionine, reveals maximum activity. The molecular weight estimated by gel-filtration was 35,000 (Fig. 4).

Increase in activity by re-aggregation of subunits has not been observed at present. Since the fused protein with maltose binding protein and subunits showed its activity, we have at present an idea that the present enzyme might be a monomer. However, the possibility that large activity can be revealed by constructing multimer, can not completely denied.

The reaction mechanism synthesizing nicotianamine from S-adenosylmethionine may be similar to methyl transfer reaction using S-adenosylmethionine as a methyl donor, and a reaction synthesizing spermidine and spermine from decarboxylated S-adenosylmethionine. The common catalytic domain of these enzymes has been discussed in relation to equivalent amino acids configuration occupying similar positions in higher-order structures (Hashimoto et al. 1998 and Schluckebier et al. 1995).

In future, catalytic domain may be elucidated as the results of comparison with nicotianamine synthase from other plant species or X-ray crystallography.

Induction of nicotianamine synthase activity by Fe-deficiency is a specific

phenomenon in graminaceous plants, and is essential for mass production of mugineic acid family. Oryza sativa is a plant, in which secretion of mugineic acid family is the least among major graminaceous plants, consequently it is very weak for Fe-deficiency in calcareous soil.

Consequently, as a result of creating transformant Oryza sativa having tolerance to Fe-deficiency by introducing nicotianamine synthase gene of the present invention into the graminaceous plants, especially Oryza sativa, and expressing large amount at the Fe-deficiency, cultivation of rice in the calcareous soil can be possible.

Heretofore, in the graminaceous plants, nicotianamine has been thought to have only a role as a precursor for synthesis of mugineic acid family. However, since the present invention has elucidated that nicotianamine synthase gene constituted the multiple gene family, it may play other important roles in the graminaceous plants.

In plants, which lack the ability to secrete mugineic acid family, except for graminaceous plants, it has been proposed that nicotianamine plays a key role as an endogenous chelator of divalent metal cations, such as Fe^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+}, and that it contributes to the homeostasis of those metals (Stephan et al. 1994). Consequently, it may play the same role in the graminaceous plants.

Nicotianamine synthase activity is not induced in dicots, and expression of gene of the present invention may not be induced by Fe-deficiency. We have cloned nicotianamine synthase genes of Arabidopsis thaliana. Composition of promoter regions in these genes can elucidate the mechanism of gene expression caused by Fe-deficiency, and the gene of the present invention may play important function not only in the graminaceous plants but also in the dicots.

SEQ ID NO: 1 shows amino acid sequence of nicotianamine synthase of the present invention.

The present invention includes nicotianamine synthase having amino acid sequence shown in SEQ ID NO: 1. However, the present invention is not limited within the above nicotianamine synthase. The nicotianamine synthase of the present invention includes, unless it loses nicotianamine synthase activity, the peptides, in which a part of the amino acid sequence of said peptide is deleted, preferably 50% or less, more preferably 30% or less, or more further preferably 10% or less in the total amino acids, or is substituted by other amino acids, or to which other amino acids are further added, or in which these deletion, substitution and addition may be combined.

Nucleotide sequence coding nicotianamine synthase of the present invention is shown in SEQ ID NO: 2.

The present invention also includes not only a gene coding nicotianamine synthase shown in SEQ ID NO: 2 but also genes coding nicotianamine synthase mentioned hereinabove.

The vector of the present invention introducing the above gene is not specifically limited, and various vectors can be introduced. Preferable vector is the expression vector.

Various cells can be transformed conventionally by using recombinant vector of the present invention. Mass production of nicotianamide can be performed by using the thus obtained transformant. These methods are well known in the person skilled in the art.

Examples of hosts for introducing the gene of the present invention are bacteria, yeasts and cells. Preferable host is plants, especially the graminaceous plant.

Method for introducing gene is not limited. It can be made by using vector or can be directly introduce in genome.

Antibody of the present invention against nicotianamine synthase can be

prepared conventionally by using nicotianamine synthase of the present invention. Antibody can be a polyclonal antibody or, if necessary, monoclonal antibody.

Further, a selective breeding of plants, preferably graminaceous plants, can be made by using gene of the present invention. Especially, the gene of the present invention can be applied for improvement of varieties, which can grow even in Fe-deficient soil.

Examples

The following examples illustrate the present invention, but are not construed as limiting the present invention.

Example 1. (Preparation of plant material)

Seeds of barley (*Hordeum vulgare* L. cv Ehimehadakamugi No. 1) were germinated on wet filter paper and transferred into the standard hydroponic culture solution (Mori and Nishizawa, 1987) in a glass house at natural temperature under natural light. The pH of the hydroponic culture solution was adjusted at 5.5 by 0.5 N HCl everyday. When the third leaves developed, the plants were transferred to the hydroponic culture solution without containing Fe. The pH was maintained at 7.0 by 0.5 N NaOH everyday. The control plants were also cultured in the standard culture solution continuously. The culture solution was renewed once in every week. Two weeks after starting Fe-deficient treatment, when severe iron chlorosis significantly appeared on the 4th and 5th leaves, roots were harvested and frozen in liquid N_2 and stored at $-80℃$ until use.

Example 2. (Assay of nicotianamine synthase activity)

Modified assay method reported previously by the present inventors (Higuchi et al. 1996a) was used. Enzyme solutions were equilibrated with reaction buffer [50 mM Tris, 1 mM EDTA, 3 mM dithiothreitol (hereinafter designates as DTT), 10μM (p-amidinophenyl) methanesulfonyl fluoride (hereinafter designates as p-APMSF) and 10μM trans-epoxysuccinyl-leucylamido-(4-guanidino) butane (hereinafter designates as E-64), pH 8.7]. Buffer exchange was performed by using ultrafiltration unit, Ultrafree C3LGC NMWL10000 (Millipore Co.). S-adenosylmethionine labeled with ^{14}C in carboxyl group (Amersham Inc.) was added to the enzyme solution at the final concentration of 20μM and kept at 25℃ for 15 minutes. The reaction products were separated by thin layer chromatography on silica gel LK6 (Whatman Inc.) using developer (phenol : butanol : formic acid : water = 12 : 3 : 2 : 3). Radioactivity of the reaction products was detected by image Analyzer BAS-2000 (Fuji Film Co.). The protein content was assayed by Bradford method using Protein Assay Kit (Bio Rad Inc.).

Example 3 (Purification of nicotianamine synthase)

The following operations were performed at 4℃ and E-64 was added to fractions containing nicotianamine synthase at the final concentration of 10μM.

The frozen roots were crushed into a fine powder in liquid N_2 and homogenized in a household juicer with 200 ml of extraction buffer [0.2 M Tris, 10 mM EDTA, 5% (v/v) glycerol, 10 mM DTT, 0.1 mM E-64, 0.1 mM p-APMSF and 5% (w/v) insoluble polyvinylpyrrolidone (PVP), pH 8.0] per 100 g of roots. The homogenate was centrifuged for 30 minutes at 22,500 × g to obtain supernatant. Ammonium sulfate was added to the supernatant to yield a final concentration of 0.4 M and allowed to stand for 1 hour. Again, the mixture was centrifuged for 30 minutes at 22,500 × g to

obtain supernatant.

The supernatant was loaded onto a TSK gel Butyl Toyopearl 650M column (10 ml bed volume per 100 g of roots), equilibrated with the adsorption buffer [20 mM Tris, 1 mM EDTA, 3 mM DTT, 0.4 M $(NH_4)_2SO_4$ and 0.1 mM p-APMSF, pH 8.0] and eluted with elution buffer [10 mM Tris, 1mM EDTA, 3 mM DTT, 0.1 mM p-APMSF, 5% glycerol and 0.05% 3-[(3-chloramidopropyl) dimethyl-ammonio] propanesulfonic acid (hereinafter designates as CHAPS), pH 8.0].

KCl was added to the active fraction to give a final concentration of 0.4 M, and 1 M potassium phosphate buffer (pH 8.0) was added to a final concentration of 1 mM of KCl. A hydroxyapatite 100 - 350 mesh (Nacalai Tesque), equilibrated with the adsorption buffer (1 mM K-P, 10 mM KCl, 3 mM DTT and 0.1 mM p-APMSF, pH 8.0), was prepared at 10 ml per protein 100 mg and the fractions containing nicotianamine synthase were loaded. Nicotianamine synthase was passed through without adsorption. The passed through fraction was loaded onto TSK gel Butyl Toyopearl 650M column (1 ml bed volume per 10 mg of protein), and nicotianamine synthase was eluted in the manner described above.

The active fraction was loaded onto a DEAE-Sepharose FF column (5 ml bed volume per 25 mg of protein, Pharmacia) equilibrated with the adsorption buffer (20 mM Tris, 1 mM EDTA, 3 mM DTT, 0.1 mM p-APMSF and 0.05% CHAPS, pH 8.0) and eluted with stepwise gradient elution of potassium chloride concentration of 0.05 M, 0.1 M, 0.15 M and 0.2 M. Nicotianamine synthase was eluted at 0.15 M of KCl concentration.

The active fraction was loaded onto the Ether Toyopearl 650M column (10 ml bed volume per 100g of roots), equilibrated with adsorption buffer [20 mM Tris, 1 mM EDTA, 3 mM DTT, 1.2 M $(NH_4)_2SO_4$ and 0.1 mM p-APMSF, pH 8.0]. Nicotianamine

synthase was not adsorbed and passed through from the column. The passed through fraction was loaded onto TSK gel Butyl Toyopearl 650M column and fractions containing nicotianamine synthase was eluted. The peptides in the active fraction containing nicotianamine synthase, which was purified by the above column chromatographic treatments, were separated by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (hereinafter designates as SDS-PAGE) using 11% acrylamide slab gels. After SDS-PAGE the gel was stained with 0.3 M copper chloride (Dzandu et al. 1988), and the separated bands were cut out. The gel fragments were destained with 0.25 M EDTA/0.25 M Tris (pH 9.0) and homogenized with the extraction buffer (1% SDS, 25 mM Tris and 192 mM glycine). Each homogenate was electroeluted with SDS-free buffer (25 mM Tris and 192 mM glycine) and peptide was recovered.

Example 4. (Determination of partial amino acid sequence)

The isolated nicotianamine synthase was digested chemically with cyanogen bromide (Gross 1967).

After SDS-PAGE treatment, 10-fold volume of 70% (v/v) formic acid and 1% (w/v) cyanogen bromide were added to gel fragments containing nicotianamine synthase and decomposed at 4℃ for overnight. After completion of digestion, the liquid part was collected and dried in vacuo. The dried substance was dissolved in SDS-PAGE sample buffer, and allowed to stand at room temperature for overnight, then the digested product was separated by SDS-PAGE using 16.5% acrylamide gel containing Tricine (Schagger and Jagow, 1987). The peptides were transferred onto a PVDF membrane by electroblotting (Towbin et al. 1979) and stained with amido black. The stained bands were cut out and the amino acid sequence was determined from N-

terminal side of each peptide by Edman degradation in gas-phase sequencer (model 492A protein sequencer, Applied Biosystems Inc.).

Example 5. (Cloning of nicotianamine synthase genes)

PCR amplification was conducted for cDNA originated from Fe-deficient barley roots using primers, which were synthesized based on the obtained partial amino acid sequence. A pYH23 cDNA library prepared from the poly $(A)^+$RNA of Fe-deficient barley roots was screened with the thus obtained DNA fragments of PCR product, which was labeled with [α-^{32}P]dATP using the random primer kit (Takara Shuzo Co.), as the primers. The isolated cDNA clones were sequenced by cycle sequencing kit (Shimadzu Bunko Co.) using Shimadzu DNA sequencer DSQ-2000L.

PCR amplification was conducted for genomic DNA of Arabidopsis thaliana using primers, which were synthesized based on nucleotide sequences of AC003114 and AB005245 of Arabidopsis thaliana. The thus obtained DNA fragments were sequenced by cycle sequencing kit (Shimadzu Bunko Co.) using Shimadzu DNA sequencer DSQ-1000L.

The determined nucleotide sequence is shown in SEQ ID NO: 2.

Example 6. (Expression of NAS1 protein in E. coli)

A fragment, in which EcoRI site was introduced into the upstream of the first ATG of the HvNAS1 cDNA and PstI and BamHI sites were introduced into the downstream of the stop codon of the HvNAS1 cDNA, was amplified by PCR. The thus obtained amplified product was subcloned in the pBluescriptII SK- using EcoRI site and BamHI site, and the correct nucleotide sequence was confirmed. The fragment between EcoRI site and PstI site was cloned into pMAL-c2 to make expression in the

form of fusing the HvNAS1 to the C-terminal of maltose binding protein.

A fragment, in which EcoRI site was introduced into the upstream of the first ATG of the OsNAS1 and HindIII site was introduced into the downstream of the stop codon of the OsNAS1, was amplified by PCR. The thus obtained amplified product was subcloned in the pBluescriptII SK- using EcoRI site and HindIII site, and the correct nucleotide sequence was confirmed. The fragment between EcoRI site and HindIII site was cloned into pMAL-c2 to make expression in the form of fusing the OsNAS1 to the C-terminal of maltose binding protein.

A fragment, in which EcoRI site was introduced into the upstream of the first ATG of the AtNAS1, AtNAS2 and AtNAS3 and XbaI site was introduced into the downstream of the stop codon of the AtNAS1, AtNAS2 and AtNAS3, was amplified by PCR. The thus obtained amplified products were subcloned in the pBluescriptII SK-, and the correct nucleotide sequences were confirmed. The fragment between EcoRI site and XbaI site was cloned into pMAL-c2 to make expression in the form of fusing the AtNAS1, AtNAS2 and AtNAS3 to the C-terminal of maltose binding proteins, respectively.

E. coli strain XL1-Blue was used as a host for expressing the said fused protein. pMAL-c2-HvNAS1 and pMAL-c2, respectively, were introduced into XL1-Blue. The thus obtained recombinant bacteria were cultured in LB medium containing ampicillin and tetracycline, each 50 μg/ml, at 37°C until the OD 600 of the culture reached 0.5. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to the final concentration of 0.3 mM, and continuously cultured at 37°C for 3 hours, and collected bacterial cells. Cells were suspended in 10 mM Tris buffer containing 0.2 M NaCl, 1 mM EDTA, 3 mM DTT and 0.1 mM E-64, pH 7.4 and frozen with liquid nitrogen. This was melted in ice water and ultrasonication for 15 seconds was repeated for 10 times.

Nicotianamine synthase activity of the thus obtained crude extract was assayed according to the method described in example 2 and the enzyme activity was confirmed.

Example 7. (Northern hybridization)

Northern hybridization of barley RNA was performed using DNA fragment, which was prepared by cutting HvNAS1 cDNA with HindIII and NotI and labeled with [α-^{32}P]dATP, as a probe. Total RNA was extracted from barley (Naito et al. 1988). The extracted RNA was separated by 1.4% agarose gel electrophoresis, and blotted onto Hybond-N$^+$ membranes (Amersham). Northern hybridization of rice RNA was performed using OsNAS1 ORF, which was labeled with [α-^{32}P]dATP, as a probe. Total RNA was extracted from rice. The extracted RNA was separated by 1.4% agarose gel electrophoresis, and blotted onto Hybond-N$^+$ membranes (Amersham). The membrane was hybridized with the probe in 0.5 M Church phosphate buffer (Church and Gilbert 1984), 1 mM EDTA, 7% (w/v) SDS with 100μg/ml salmon sperm DNA at 65℃ for overnight. The membrane was washed with buffer containing 40 mM Church phosphate buffer and 1% (w/v) SDS at 65℃ for 10 minutes. After the washing was repeated once again, the membrane was washed with buffer containing 0.2 × SSPE and 0.1% (w/v) SDS at 65℃ for 10 minutes. Radioactivity was detected using the image analyzer BAS-2000.

Results are shown in Fig. 9 and Fig. 16.

Example 8. (Southern hybridization)

Genomic DNA was extracted from leaves of barley and rice. The extract was digested with BamHI, EcoRI or HindIII, separated on a 0.8% (w/v) agarose gel

electrophoresis, and transferred onto Hybond-N$^+$ membranes (Amersham). The hybridization was performed according to the method described in example 7 and radioactivity was detected.

Result is shown in Fig. 10.

Example 9 (Preparation of polyclonal antibody)

Total protein was extracted using trichloroacetic acid and acetone (Damerval et al. 1986). The plants were crashed in the liquid nitrogen until powder was obtained, and mixed with acetone containing 0.1% (v/v) 2-mercaptoethanol. The protein was precipitated by allowing to stand at –20℃ for 1 hour, and the precipitate was collected by centrifugation at 16,000 × g for 30 minutes. The precipitate was suspended in acetone containing 0.1% (v/v) 2-mercaptoethanol and allowed to stand at –20℃ for 1 hour, then collected the precipitate by centrifugation at 16,000 × g for 30 minutes. The precipitate was dried in vacuo, and dissolved in the sample buffer [9.5 M urea, 2% (w/v) Triton X-100 and 5% (v/v) 2-ME], then centrifuged at 16,000 × g for 10 minutes to obtain the supernatant. The proteins contained in the supernatant were separated by SDS-PAGE or the denaturing two-dimensional electrophoresis (O'Farrell 1975) and transferred onto PVDF membrane. Western blotting analysis was performed by applying the primary antibody containing anti-nicotianamine synthase antibody prepared in example 1 and the secondary antibody containing horse radish binding anti-mouse IgG (H + L) goat antibody (Wako Pure Chemicals Co.) on the membrane and coloring with diaminobenzidin.

Result is shown in Fig. 12. SDS-PAGE was performed using 12.5% acrylamide slab gel. Protein 100μg was electrophoresed. Proteins of roots 200μg and leaves 500 μg were electrophoresed.

Example 11 (RT-PCR)

Total RNA was extracted from Arabidopsis thaliana. RT-PCR was performed with 1 μg RNA as a template by using the EZ rTth RNA PCR kit (Parkin Elmer Inc.). Specific primers for AtNAS1, AtNAS2 and AtNAS3, respectively, were used.

Result is shown in Fig. 18.

Industrial Applicability

Various cells are transformed according to the conventional method by using recombinant vectors of the present invention. Mass production of nicotianamine can be performed by using the obtained transformant. These methods can be performed according to the method known in the person skilled in the art.

Selective breeding of plants, preferably graminaceous plants can also be performed using genes of the present invention. Especially, genes of the present invention can be applied for improving varieties, which can grow on Fe-deficient soil.

CLAIMS

1. A nicotianamine synthase comprising amino acid sequence shown in SEQ ID NO: 1, or amino acid sequence having deletion in a part thereof, being substituted by the other amino acids or being added with the other amino acids.

2. The nicotianamine synthase according to claim 1 wherein said enzyme is originated from barley.

3. The nicotianamine synthase according to claim 1 or 2 comprising having amino acid sequence shown in SEQ ID NO: 1, 3, 5, 7, 9, 11 or 13.

4. The nicotianamine synthase according to claim 1 wherein said enzyme is originated from Arabidopsis.

5. The nicotianamine synthase according to claim 1 or 4 comprising having amino acid sequence shown in SEQ ID NO: 17, 19 or 21.

6. The nicotianamine synthase according to claim 1 wherein said enzyme is originated from Oryza sativa.

7. The nicotianamine synthase according to claim 1 or 6 comprising having amino acid sequence shown in SEQ ID NO: 15.

8. A gene encoding amino acid sequence of nicotianamine synthase according to any one of claims 1 - 7.

9. The gene according to claim 8 wherein said gene is cDNA.

10. The gene according to claim 8 or 9 comprising having base sequence shown in SEQ ID NO: 2, 4, 6, 8, 10, 12 or 14.

11. The gene according to claim 8 or 9 comprising having base sequence shown in SEQ ID NO: 18, 20 or 22.

12. A vector comprising containing gene according to any one of claims 8 - 11.

13. The vector according to claim 12 wherein said vector is an expression vector.

14. A transformant wherein said transformant is transformed by the vector according to claim 12 or 13.

15. The transformant according to claim 14 wherein the foreign gene is a gene having base sequence shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, or 22.

16. The transformant according to claim 14 or 15 wherein the host is bacteria.

17. The transformant according to claim 14 or 15 wherein the host is higher bacteria.

18. A process for production of nicotianamine comprising using the transformant according to any one of claims 14 - 17.

19. A plant wherein the gene according to any one of claims 8 - 10 is introduced.

20. The plant according to claim 19 wherein said plant is seed.

21. A fruit obtained by growing the plant according to claim 19 or 20.

22. An antibody against nicotianamine synthase according to any one of claims 1 - 7.

23. The antibody according to claim 22 wherein said antibody is polyclonal antibody.

24. The antibody according to claim 22 wherein said antibody is monoclonal antibody.

25. A method for extraction of nicotianamine synthase comprising extracting the said enzyme in the presence of thiol protease inhibitor at the extraction of nicotianamine synthase from the plant.

26. The method according to claim 25 wherein the thiol protease inhibitor is E-64.

Fig. 1

Fig. 2

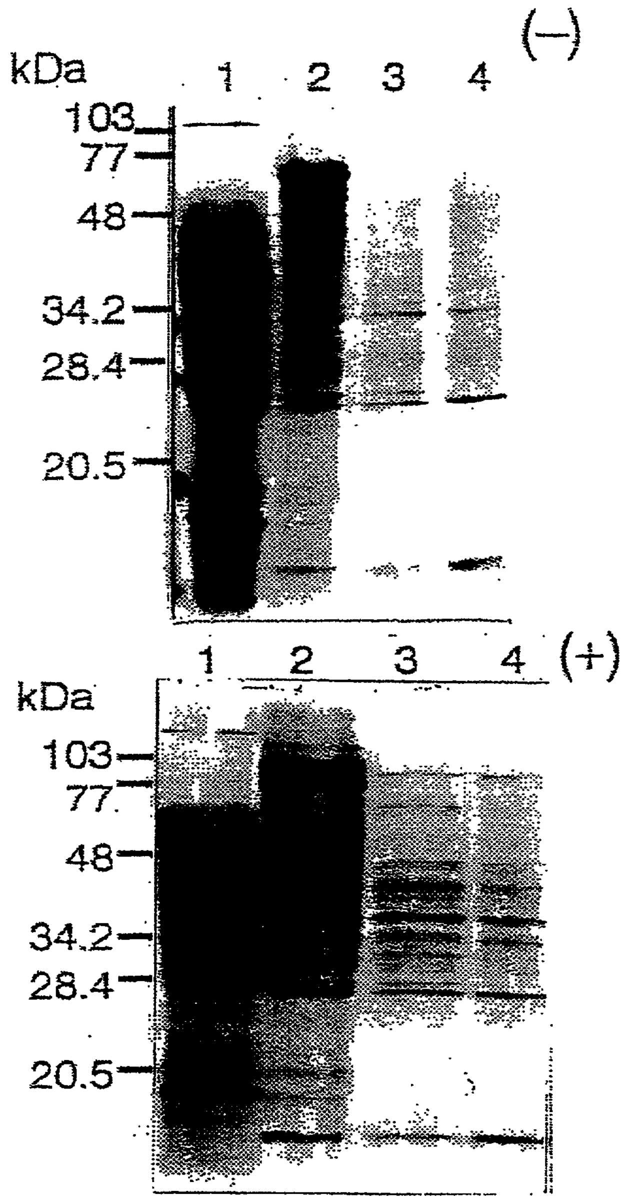


Fig. 3

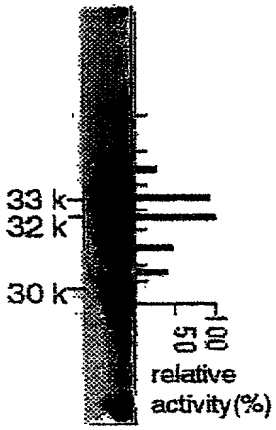
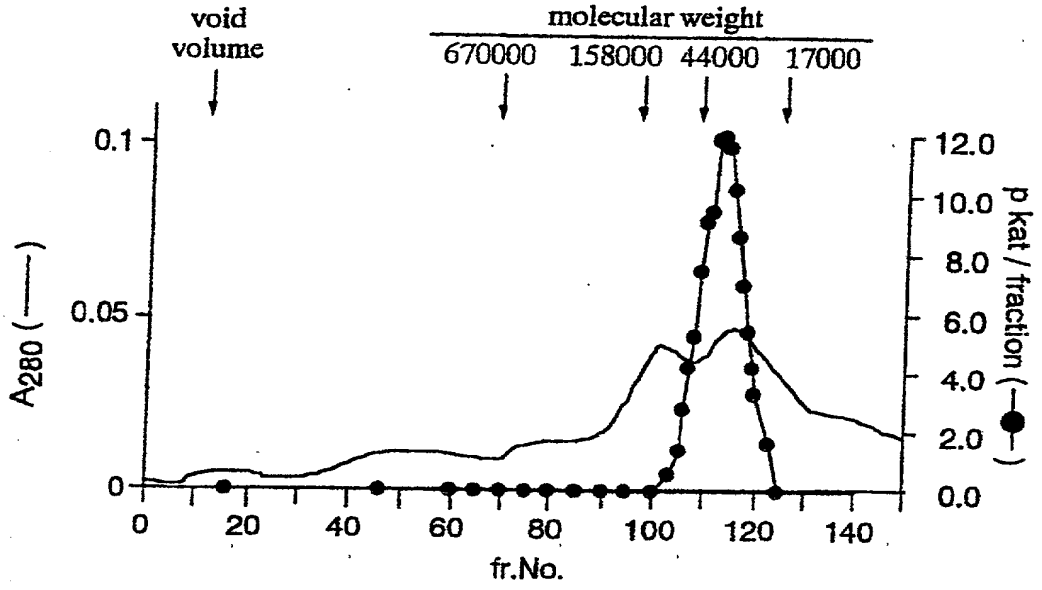


Fig. 4


void volume
molecular weight
670000
158000
44000
17000
0.1
0.05
0
A_{280} (——)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
p kat / fraction (—●—)
0
20
40
60
80
100
120
140
fr.No.

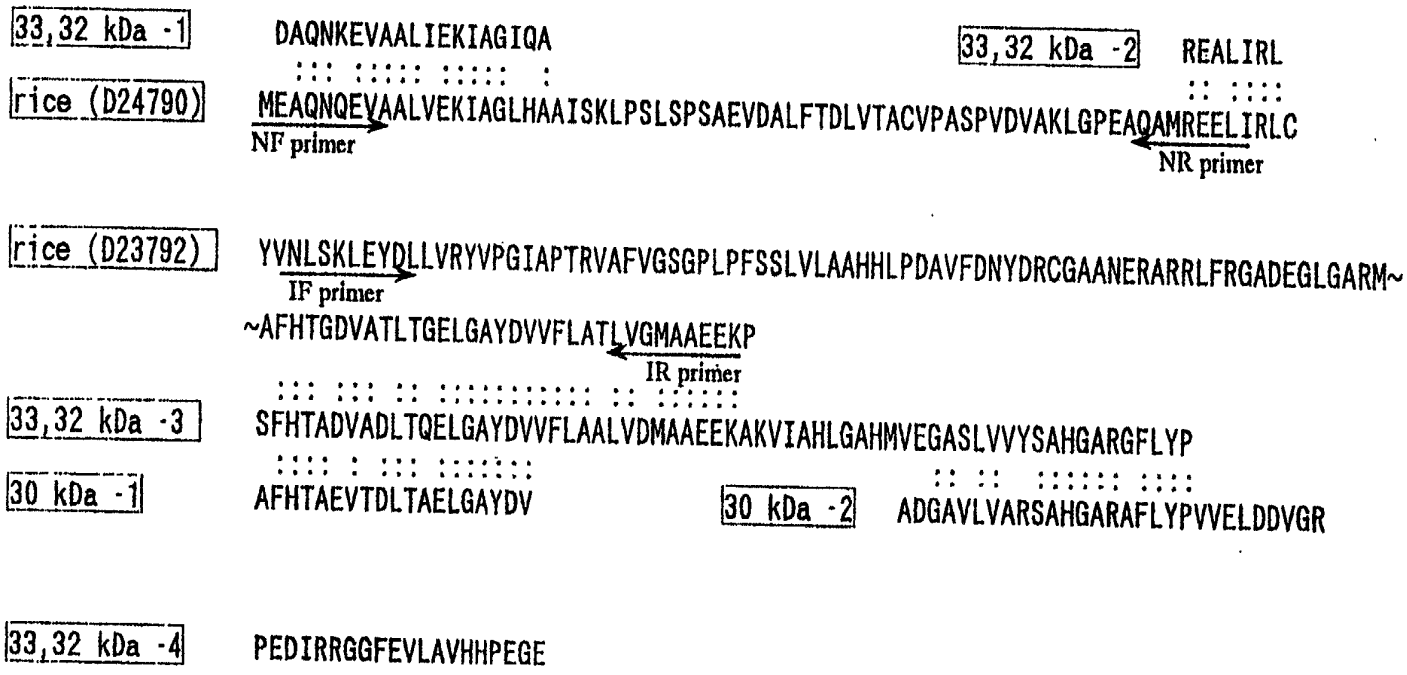


Fig. 5

Fig. 6

```
     GCG TTC AGA GGC TTC CAG AGT TCT TCC GGT CAC CAA GAA GCA TTT GAT CAT AAC    54

     ATG GAT GCC CAG AAC AAG GAG GTC GCT GCT CTG ATC GAG AAG ATC GCC GGT ATC   108
19   M  D  A  Q  N  K  E  V  A  A  L  I  E  K  I  A  G  I
     (1)

     CAG GCC GCC ATC GCC GAG CTG CCG TCG CTG AGC CCG TCC CCC GAG GTC GAC AGG   162
37   Q  A  A  I  A  E  L  P  S  L  S  P  S  P  E  V  D  R

     CTC TTC ACC GAC CTC GTC ACG GCC TGC GTC CCG CCG AGC CCC GTC GAC GTG ACG   216
55   L  F  T  D  L  V  T  A  C  V  P  P  S  P  V  D  V  T

     AAG CTC AGC CCG GAG CAC CAG AGG ATG CGG GAG GCT CTC ATC CGC TTG TGC TCC   270
73   K  L  S  P  E  H  Q  R  M  R  E  A  L  I  R  L  C  S
                               (2)

     GCC GCC GAG GGG AAG CTC GAG GCG CAC TAC GCC GAC CTG CTC GCC ACC TTC GAC   324
91   A  A  E  G  K  L  E  A  H  Y  A  D  L  L  A  T  F  D

     AAC CCG CTC GAC CAC CTC GGC CTC TTC CCG TAC TAC AGC AAC TAC GTC AAC CTC   378
109  N  P  L  D  H  L  G  L  F  P  Y  Y  S  N  Y  V  N  L

     AGC AGG CTG GAG TAC GAG CTC CTG GCG CGC CAC GTG CCG GGC ATC GCG CCG GCG   432
127  S  R  L  E  Y  E  L  L  A  R  H  V  P  G  I  A  P  A

     CGC GTC GCC TTC GTC GGC TCC GGC CCG CTG CCG TTC AGC TCG CTC GTC CTC GCC   486
145  R  V  A  F  V  G  S  G  P  L  P  F  S  S  L  V  L  A

     GCG CAC CAC CTG CCC GAG ACC CAG TTC GAC AAC TAC GAC CTG TGC GGC GCG GCC   540
163  A  H  H  L  P  E  T  Q  F  D  N  Y  D  L  C  G  A  A

     AAC GAG CGC GCC AGG AAG CTG TTC GGC GCG ACG GCG GAC GGC GTC GGC GCG CGT   594
181  N  E  R  A  R  K  L  F  G  A  T  A  D  G  V  G  A  R

     ATG TCG TTC CAC ACG GCG GAC GTC GCC GAC CTC ACC CAG GAG CTC GGC GCC TAC   648
199  M  S  F  H  T  A  D  V  A  D  L  T  Q  E  L  G  A  Y
     (3)

     GAC GTG GTC TTC CTC GCC GCG CTC GTC GGC ATG GCA GCC GAG GAG AAG GCC AAG   702
217  D  V  V  F  L  A  A  L  V  G  M  A  A  E  E  K  A  K

     GTG ATT GCC CAC CTG GGC GCG CAC ATG GTG GAG GGG GCG TCC CTG GTC GTG CGG   756
235  V  I  A  H  L  G  A  H  M  V  E  G  A  S  L  V  V  R

     AGC GCA CGG CCC CGC GGC TTT CTT TAC CCC ATT GTC GAC CCG GAG GAC ATC AGG   810
253  S  A  R  P  R  G  F  L  Y  P  I  V  D  P  E  D  I  R
                                   (4)

     CGG GGT GGG TTC GAG GTG CTG GCC GTG CAC CAC CCG GAA GGT GAG GTG ATC AAC   864
271  R  G  G  F  E  V  L  A  V  H  H  P  E  G  E  V  I  N

     TCT GTC ATC GTC GCC CGT AAG GCC GTC GAA GCG CAG CTC AGT GGG CCG CAG AAC   918
289  S  V  I  V  A  R  K  A  V  E  A  Q  L  S  G  P  Q  N

     GGA GAC GCG CAC GCA CGG GGC GCG GTG CCG TTG GTC AGC CCG CCA TGC AAC TTC   972
307  G  D  A  H  A  R  G  A  V  P  L  V  S  P  P  C  N  F

     TCC ACC AAG ATG GAG GCG AGC GCG CTT GAG AAG AGC GAG GAG CTG ACC GCC AAA  1026
325  S  T  K  M  E  A  S  A  L  E  K  S  E  E  L  T  A  K

     GAG CTG GCC TTT TGA TTG AAG AGT GCG CGT GGT CAT TCT GTC GCC TGC GAT CGT  1080
     E  L  A  F  *

     GGT AAC TTT CCT ACT CGT GTG TGT TTT GAT GTT TGT GCC TGT AAG AGT TAT GCT  1134
     TCC GGC CTT GTG CTG TTA ATT TAC ACG CGT TAC ATG TAG TAC TTG TAT TTA TAC  1188
     CTG GAA TAA CGG TAT GTA ACA TAA ATA TTA GTG GGA TTT GAA GTG TAA TGC TAA  1242
     ATA ATA AGA AAA CTT GAT GCA GAC ATT CAA AAA AAA AAA AAA AAA AAA AAA AA
```

Fig. 7

```
HvNAS4      MDGQSE--EVDALVQKITGLHAAIAKLPSLSPSPDVDALFTDLVTACVPPSPVDVTKLAP
HvNAS7      MDAQSK--EVDALVQKITGLHAAIAKLPSLSPSPDVDALFTDLVTACVPPSPVDVTKLAP
HvNAS6      MDAQNK--EVDALVQKITGLHAAIAKLPSLSPSPDVDALFTDLVTACVPPSPVDVTKLGS
HvNAS2      MAAQNN-QEVDALVEKITGLHAAIAKLPSLSPSPDVDALFTELVTACVPPSPVDVTKLGP
HvNAS3      MAAQNNNKDVAALVEKITGLHAAIAKLPSLSPSPDVDALFTELVTACVPPSPVDVTKLGP
HvNAS1      MDAQNK--EVAALIEKIAGIQAAIAELPSLSPSPEVDRLFTDLVTACVPPSPVDVTKLSP
HvNAS5      MEAENG--EVAALVEKITGLHAAISKLPALSPSPQVDALFTELVAACVPSSPVDVTKLGP
            *        * **  ** *  ***  ** ***** ** *** ** **** ********

HvNAS4      EAQAMREGLIRLCSEAEGKLEAHYSDMLAAFDNPLDHLGVFPYYSNYINLSKLEYELLAR
HvNAS7      EAQAMREGLIRLCSEAEGKLEAHYSDMLAAFDNPLDHLGVFPYYSNYINLSKLEYELLAR
HvNAS6      EAQEMREGLIRLCSEAEGKLEAHYSDMLAAFDNPLDHLGMFPYYSNYINLSKLEYELLAR
HvNAS2      EAQEMREGLIRLCSEAEGKLEAHYSDMLAAFDKPLDHLGMFPYYNNYINLSKLEYELLAR
HvNAS3      EAQEMREGLIRLCSEAEGKLEAHYSDMLAAFDNPLDHLGIFPYYSNYINLSKLEYELLAR
HvNAS1      EHQRMREALIRLCSAAEGKLEAHYADLLATFDNPLDHLGLFPYYSNYVNLSRLEYELLAR
HvNAS5      EAQEMRQDLIRLCSAAEGLLEAHYSDMLTALDSPLDHLGRFPYYFDNYVNLSKLEHDLLAG
            * * **  ****** *** ***** * *   * ****** ***  ** *** **  ***

HvNAS4      YVPGRHRPARVAFIGSGPLPFSSYVLAARHLPDTVFDNYDLCGAANDRATRLFRADKD-V
HvNAS7      YVPGGIAPARVAFIGSGPLPFSSYVLAARHLPDTVFDNYVPVRAANDRATRLFRADKD-V
HvNAS6      YVPGGIARPAVAFIGSGPLPFSSYVLAARHLPDAMFDNYDLCSAANDRASKLFRADKD-V
HvNAS2      YVPGGYRPARVAFIGSGPLPFSSFVLAARHLPDTMFDNYDLCGAANDRASKLFRADRD-V
HvNAS3      YVRR-HRPARVAFIGSGPLPFSSFVLAARHLPDTMFDNYDLCGAANDRASKLFRADTD-V
HvNAS1      HVPG-IAPARVAFVGSGPLPFSSLVLAAHHLPETQFDNYDLCGAANERARKLFGATADGV
HvNAS5      HVAA---PARVAFIGSGPLPFSSLFLATYHLPDTRFDNYDRCSVANGRAMKLVGAADEGV
             *        *** *********  **  ***   ****     ** **  * *    *

HvNAS4      GARMSFHTADVADLTDELATYDVVFLAALVGMAAEDKAKVIAHLGAHMADGAALV--ARH
HvNAS7      GARMSFHTADVADLTDELATYDVVFLAALVGMAAEDKGQGDPHLGAHMADGAALVR-SAH
HvNAS6      GARMSFHTADVADLTRELAAYDVVFLAALVGMAAEDKAKVIPHLGAHMADGAALVV-RSA
HvNAS2      GARMSFHTADVADLAGELAKYDVVFLAALVGMAAEDKAKVIAHLGAHMADGAALVVRSAH
HvNAS3      GARMSFHTADVADLASELAKYDVVFLAALVGMAAEDKAKVIAHLGAHMADGAALVVRSAH
HvNAS1      GARMSFHTADVADLTQELGAYDVVFLAALVGMAAEEKAKVIAHLGAHMVEGASLVV-RSA
HvNAS5      RSRMAFHTAEVTDLTAELGAYDVVFLAALVGMTSKEKADAIAHLGKHMADGAVLVREALH
              ** **** * **  **  ************    *     *** **  ** **

HvNAS4      GARGFLYPIVDPQDIGRGGFEVLAVCHPD-DDVVNSVIIAQKSNDVHEYGLGSGR--GGR
HvNAS7      GARGFLYPIVDPQDIGRGGFEVLAVCHPD-DDVVNSVIIAQKSKDMFANGPRNGC--GGR
HvNAS6      QARGFLYPIVDPQDIGRGGFEVLAVCHPD-DDVVNSVIIAHKSKDVHANERPNGR--GGQ
HvNAS2      GARGFLYPIVDPQDIGRGGFEVLAVCHPD-DDVVNSVIIAQKSKDVHADGLGSGRGAGGQ
HvNAS3      GARGFLYPIVDPQDIGRGGFEVLAVCHPD-DDVVNSVIIAQKSKEVHADGLGSARGAGRQ
HvNAS1      RPRGFLYPIVDPEDIRRGGFEVLAVHHPE-GEVINSVIVARKAVEAQLSGPQNGD----A
HvNAS5      GARAFLYPVVELDDVGRGGFQVLAVHHPAGDEVFNSFIVARKVKMSA------------
              * **** *   *  **** **** **    * ** * * *

HvNAS4      YARGTVVPVVSPPCRFG-EMVADVTQ--KREEFANAEVAF
HvNAS7      YARG-TVPVVSPPCRFG-EMVADVTQ--KREEFAKAEVAF
HvNAS6      YRGA--VPVVSPPCRFG-EMVADVTH--KREEFTNAEVAF
HvNAS2      YARG-TVPVVSPPCRFG-EMVADVTQNHKRDEFANAEVAF
HvNAS3      YARG-TVPVVSPPCRFG-EMVADVTQNHKRDEFANAEVAF
HvNAS1      HARG-AVPLVSPPCNFSTKMEASALE--KSEELTAKELAF
                  ** ***** *   * *      *  *    * **
```

Fig. 8

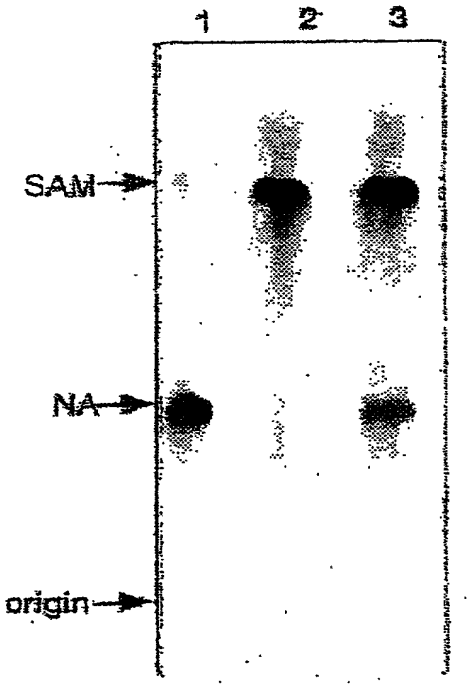


Fig. 9

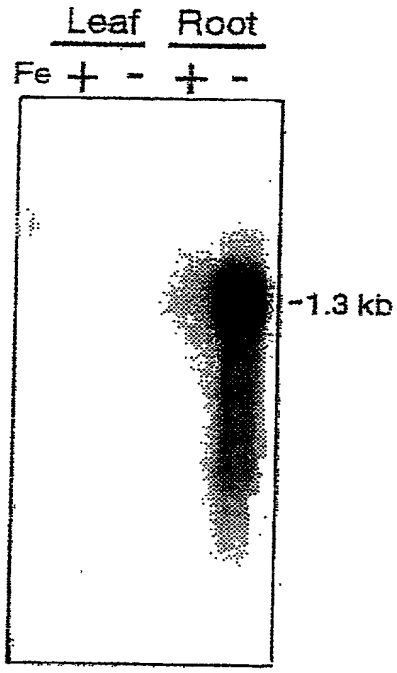


Fig. 10

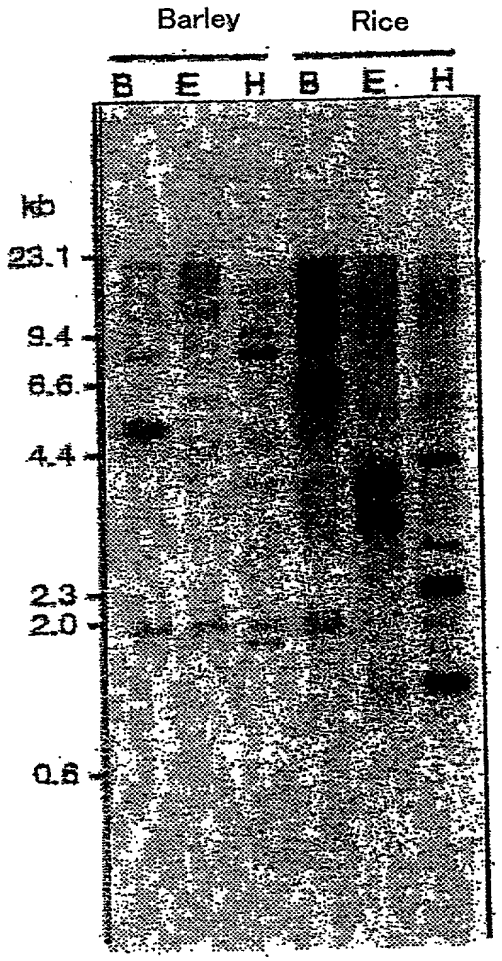


Fig. 11

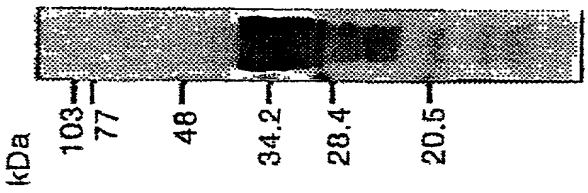


Fig. 12

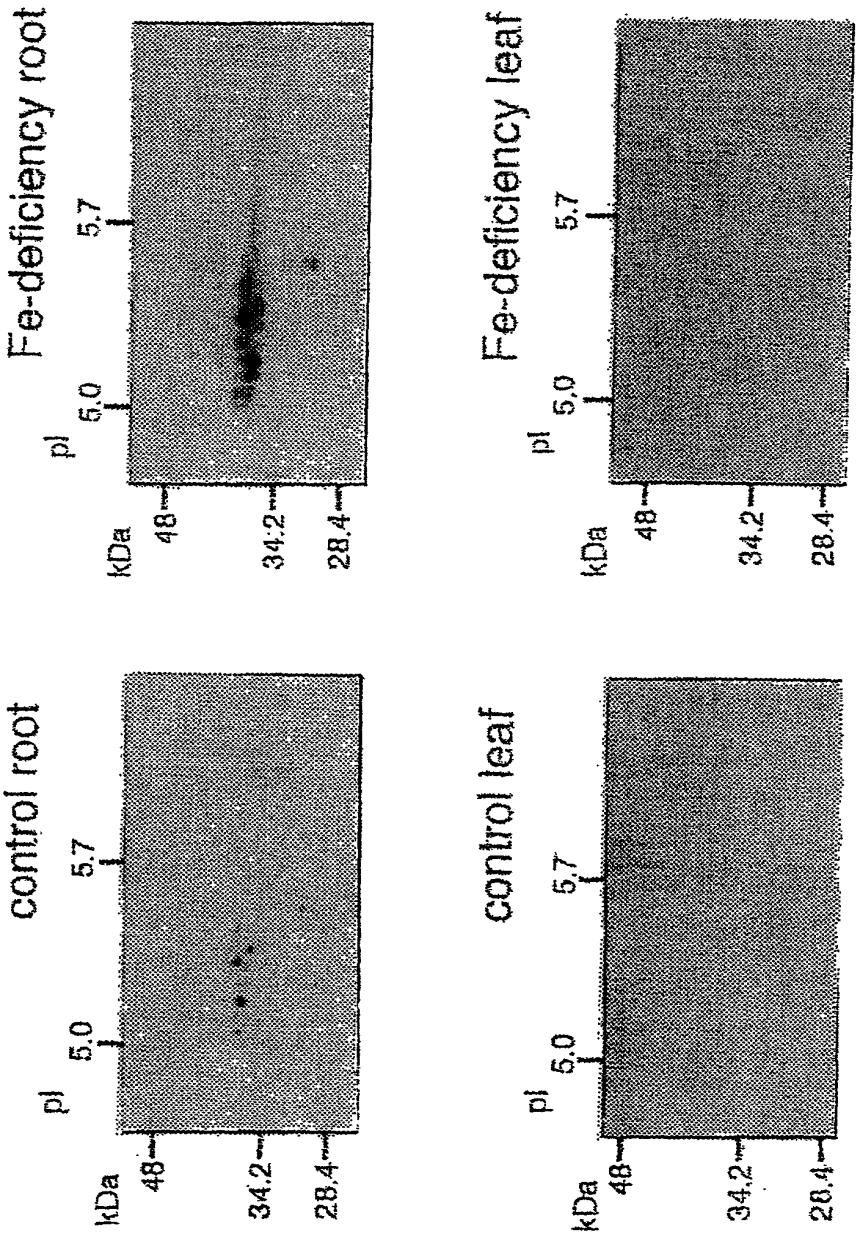


Fig. 13

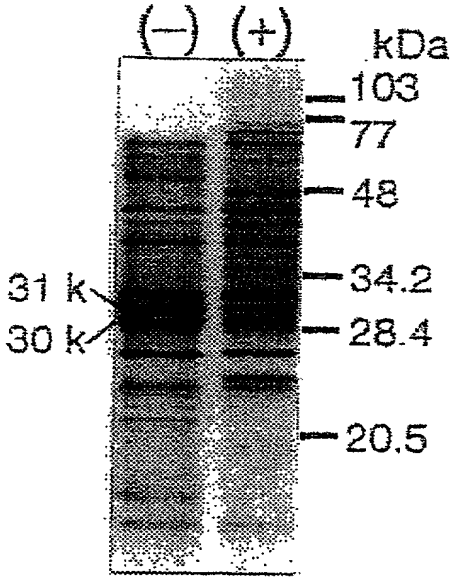


Fig. 14

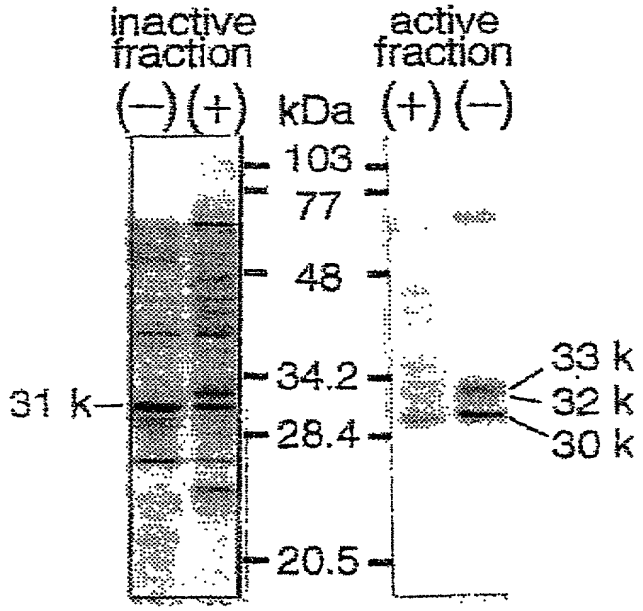

inactive fraction
active fraction
(−) (+)
kDa
(+) (−)
103
77
48
34.2
28.4
20.5
31 k
33 k
32 k
30 k

Fig. 15

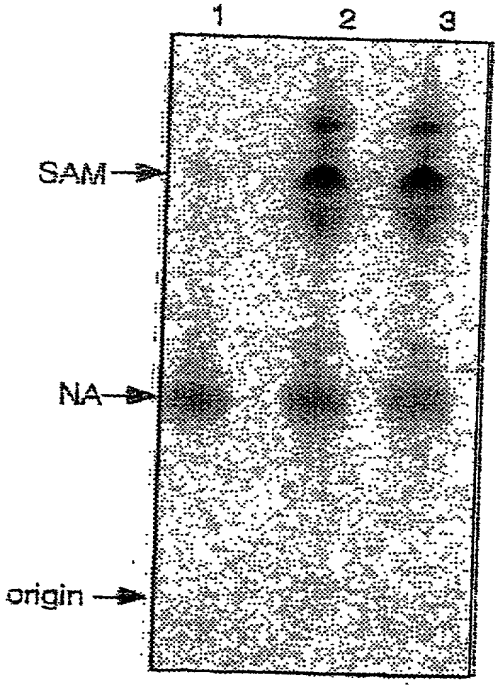


Fig. 16

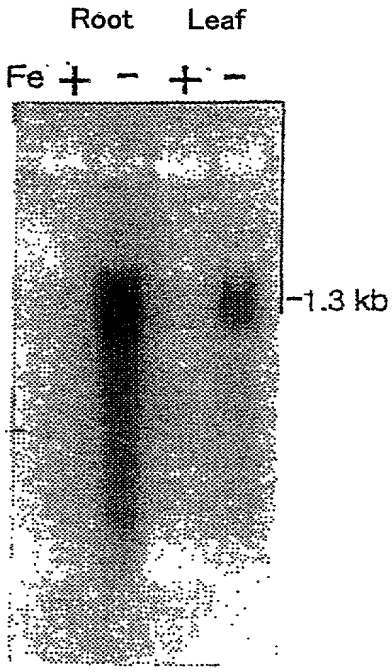


Fig. 17

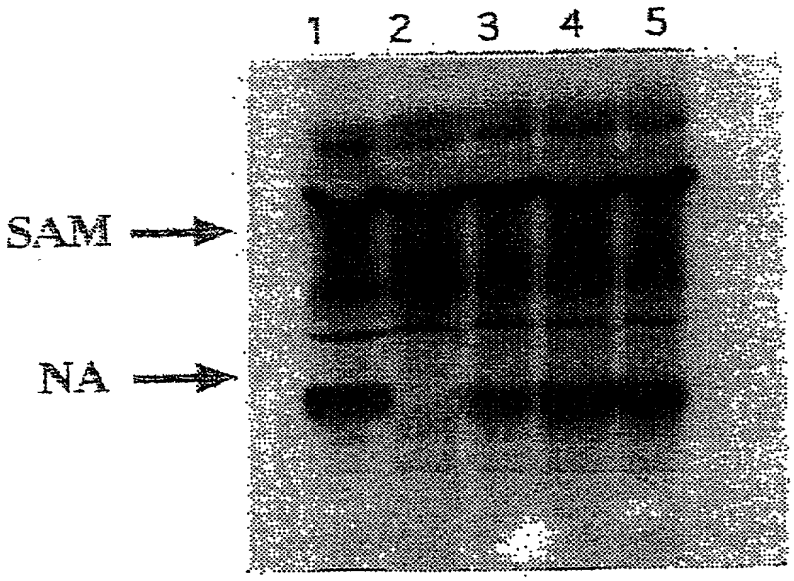
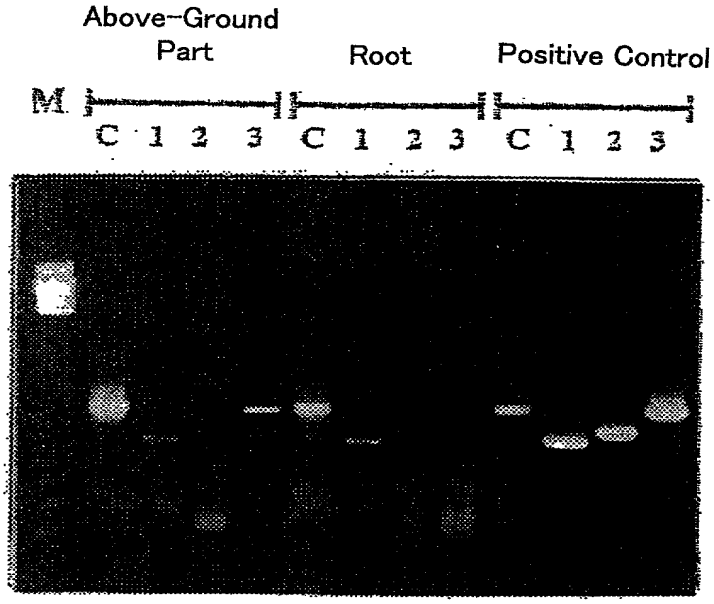


Fig. 18



55107
PP9045'

DIKE, BRONSTEIN, ROBERTS & CUSHMAN
INTELLECTUAL PROPERTY GROUP OF
EDWARDS & ANGELL, LLP
130 Water Street
Boston, Massachusetts 02109

Docket No. 55107

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that: My residence, post office address and citizenship are as stated below next to my name. I believe I am an original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled:

NICOTIANAMINE SYNTHASE AND GENE ENCODING THE SAME

which is described and claimed in:

☐ the specification attached hereto.

☒ the specification in the U.S. patent application no. **09/674,337** filed on **October 30, 2000.**

☐ the specification in PCT international application Number, filed on _____; and was amended on _____.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a). I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

Prior Foreign/PCT Applications and Any Priority Claims Under 35 U.S.C. §119:

Application No.	Filing Date	Country	Priority Claimed Under 35 U.S.C. §119?
PCT/JP99/02305	April 30, 1999	PCT	☒YES ☐NO
10-137685/1988	April 30, 1998	Japan	☒YES ☐NO
			☐YES ☐NO

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below, and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose material information as defined in 37 CFR §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

Prior U.S. Applications or PCT International Applications Designating the U.S-Benefit Under 35 U.S.C. §120				
U.S. Applications		Status (Check One)		
Application Serial No.	U.S. Filing Date	Patented	Pending	Abandoned
PCT Applications Designating the U.S.				
Application No.	Filing Date	U.S. Serial No. Assigned		

CLAIM FOR BENEFIT OF PRIOR U.S. PROVISIONAL APPLICATION(S)
(35 U.S.C. §119(e))

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below:

Applicant	Provisional Application Number	Filing Date

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) with full powers of association, substitution and revocation to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

David G. Conlin	(Reg. No. 27,026)	Cara Z. Lowen	(Reg. No. 38,227)	David A. Tucker	(Reg. No. 27,840)
George W. Neuner	(Reg. No. 26,964)	William J. Daley, Jr.	(Reg. No. 35,487)	George W. Hartnell, III	(Reg. No. 42,639)
Linda M. Buckley	(Reg. No. 31,003)	Robert L. Buchanan	(Reg. No. 40,927)	Peter F. Corless	(Reg. No. 33,860)
Christine C. O'Day	(Reg. No. 38,256)	Steven M. Jensen	(Reg. No. 42,693)	Stuart L. Gitler	(Reg. No. 31,256)
Peter J. Manus	(Reg. No. 26,766)	Lisa Hazzard Swiszcz	(Reg. No. 44,368)	Kathryn A. Piffat	(Reg. No. 34,901)

SEND CORRESPONDENCE TO:	DIRECT TELEPHONE CALLS TO:
Peter F. Corless Dike, Bronstein, Roberts & Cushman Intellectual Property Practice Group Edwards & Angell, LLP 130 Water Street Boston, Massachusetts 02109	Peter F. Corless (617) 523-3400

1-00	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
		MORI	Satoshi	
201	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
		Chiba-ken	Japan JPX	Japan
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE OR COUNTRY AND ZIP CODE
		6-7-2-301, Yatsu, Narashino-shi	Chiba-ken 275-0026	Japan
2-00	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
		HIGUCHI	Kyoko	
202	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
		Gunma	Japan JPX	Japan
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE OR COUNTRY AND ZIP CODE
		4705-1-102, Kuragaya-cho, Takasaki-shi	Gunma 370-1201	Japan
3-00	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
		SUZUKI	Kazuya	
203	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
		Tokyo	Japan JPX	Japan
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE OR COUNTRY AND ZIP CODE
		1-1-1, Yayoi, Bunkyo-ku	Tokyo 113-0032	Japan
4-00	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
		NISHIZAWA	Naoko	
204	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
		Tokyo	Japan JPX	Japan
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE OR COUNTRY AND ZIP CODE
		1-37-9-705, Hakusan, Bunkyo-ku	Tokyo 113-0001	Japan
5-00	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
		NAKANISHI	Hiromi	
205	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
		Tokyo	Japan JPX	Japan
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE OR COUNTRY AND ZIP CODE
		1-1-1, Yayoi, Bunkyo-ku	Tokyo 113-0032	Japan
	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
206	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE OR COUNTRY AND ZIP CODE

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signature of Inventor **201** Satoshi Mori **Satoshi MORI** Date: July 11, 2001	Signature of Inventor **202** Kyoko Higuchi **Kyoko HIGUCHI** Date: July 11, 2001
Signature of Inventor **203** Kazuya Suzuki **Kazuya SUZUKI** Date: July 11, 2001 Kazuya Suzuki	Signature of Inventor **204** Naoko Nishizawa **Naoko NISHIZAWA** Date: July 11, 2001
Signature of Inventor **205** Hiromi Nakanishi **Hiromi NAKANISHI** Date: July 11, 2001	Signature of Inventor **206** Date:

United States Patent & Trademark Office

Office of Initial Patent Examination -- Scanning Division

Application deficiencies found during scanning:

☐ Page(s)__________ of ______________________________ were not present for scanning. (Document title)

☐ Page(s)__________ of ______________________________ were not present for scanning. (Document title)

☑ ***Scanned copy is best available.*** Figures are dark.